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LTBP1 promotes the progression of triple negative breast cancer via activating the RhoA/ROCK signaling pathway

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ABSTRACT

The latent transforming growth factor-beta (TGF-β) binding protein 1 (LTBP1) has been implicated in various cellular processes, but its role in triple-negative breast cancer (TNBC) remains unclear. In this study, we investigated the impact of LTBP1 on TNBC progression and its underlying mechanisms. Analysis of online datasets revealed elevated LTBP1 mRNA expression in breast cancer tissues compared to normal adjacent tissues. Kaplan-Meier Plotter analysis indicated that high LTBP1 expression was negatively correlated with relapse-free survival (RFS), distant-metastasis free survival (DMFS), and overall survival (OS) of breast cancer patients. Additionally, LTBP1 mRNA levels were associated with chemotherapy resistance. Functional assays in TNBC cells demonstrated that LTBP1 knockdown suppressed cell proliferation, induced apoptosis, and attenuated migration and invasion. In vivo studies confirmed that LTBP1 knockdown inhibited tumor growth in a xenograft mouse model. Mechanistically, LTBP1 positively correlated with genes involved in signaling regulation and organelle organization, with significant associations to GTPase binding and the RhoA/ROCK pathway. LTBP1 knockdown reduced RhoA activity and phosphorylation of Myosin Light Chain 2 (MLC2), suggesting inhibition of the RhoA/ROCK signaling pathway. Moreover, activation of the RhoA/ROCK pathway partially rescued the effects of LTBP1 knockdown on TNBC cell proliferation, apoptosis, migration, and invasion. In conclusion, our findings suggest that LTBP1 promotes TNBC progression through activation of the RhoA/ROCK signaling pathway, highlighting its potential as a therapeutic target for TNBC.

KEYWORDS

LTBP1; Triple negative breast cancer; RhoA; ROCK; GTPase

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1. Introduction

Triple-negative breast cancer (TNBC) represents a subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification [1, 2]. This subtype is associated with aggressive clinical behavior, limited therapeutic options, and poor prognosis. Understanding the molecular mechanisms driving TNBC progression is crucial for the development of targeted therapeutic strategies.

In this context, the latent transforming growth factor-beta (TGF-β) binding protein 1 (LTBP1) has emerged as a potential regulator in various cellular processes [3]. LTBP1 is a member of the LTBP family, known for its role in regulating the bioavailability of TGF-β [4]. Recent studies have implicated LTBP1 in diverse cellular functions, including cell proliferation, migration, and apoptosis, suggesting its potential as a key player in tumorigenesis [3, 5]. While the involvement of TGF-β signaling in cancer is well-established, the specific contribution of LTBP1 in breast cancer, particularly in TNBC, remains poorly characterized.

Given the critical need to elucidate the molecular mechanisms driving TNBC, this study aims to investigate the role of LTBP1 in the progression of TNBC and to decipher the underlying signaling pathways. We hypothesize that LTBP1 may play a significant role in promoting TNBC aggressiveness, potentially through the activation of specific signaling cascades as the previous study has shown that LTBP1 could promote fibrillin incorporation into the extracellular matrix, which is critical in cancer metastasis [5]. Fibrillin-1 is the major component of extracellular matrix microfibrils, which are required for proper development of elastic tissues, including the heart and lungs [6]. Consistently, LTBP1 has been shown to facilitate the secretion of Fibrillin-1 [7]. Additionally, it can promote tumor growth under hypoxia by facilitating the compartmentalized LTBP1 S-Nitrosylation [8]. To address our hypothesis, we employed a comprehensive approach, combining in silico analysis of online datasets, *in vitro* experiments using TNBC cell lines, and *in vivo* studies in a xenograft mouse model.

Our initial analysis of online datasets revealed elevated expression of LTBP1 in breast cancer tissues compared to normal adjacent tissues. Kaplan-Meier survival analysis indicated a negative correlation between LTBP1 expression and relapse-free survival (RFS), distant-metastasis free survival (DMFS), and overall survival (OS) in breast cancer patients. Furthermore, LTBP1 expression showed an association with chemotherapy resistance, suggesting its potential as a predictive marker for treatment response. Functional assays in TNBC cell lines demonstrated that LTBP1 knockdown inhibited cell proliferation, induced apoptosis, and attenuated migration and invasion. *In vivo* studies confirmed the inhibitory effects of LTBP1 knockdown on tumor growth in a xenograft mouse model. Mechanistic insights revealed a positive correlation between LTBP1 and genes involved in the RhoA/ROCK signaling pathway, a pathway implicated in cellular processes such as migration, invasion, and cytoskeletal reorganization.

2. Materials and methods

2.1. Analysis of online datasets

Online dataset analysis (TNMplot: differential gene expression analysis in Tumor, Normal, and Metastatic tissues) was used to analyze the expression of genes in tumors and corresponding adjacent normal tissues, and the expression correlation of different genes [9]. Gene ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for these correlated genes were identified through this online analytic tool. Kaplan-Meier Plotter analysis (http://kmplot.com/analysis/index.php?p=background) was used to evaluate the correlation between genes and different types of survivals of tumor patients [10]. ROC Plotter - Online ROC analysis (https://www.rocplot.com/) was used to determine the correlation between gene expression and drug sensitivity [11].

2.2. Cell culture

Human breast cancer cell lines (MCF-7, T47D, BT474, MDA-MB-231, MDA-MB-453) and normal mammary epithelial cell line MCF-10A were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI1640 medium (KeyGEN BioTECH, Nanjing, China), supplemented with 10% FBS (Fetal bovine serum, Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37˚C humidified incubator supplemented with 5% CO_2.

2.3. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from the cell with RNAiso Plus reagent (Takara, Japan). cDNA was generated from 500 ng of RNA using the PrimeScript RT reagent kit. 2 μL of cDNA was subsequently used for RT-qPCR with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and the SYBR Premix Ex Tag. The conditions for RT-qPCR were as follows: pre-denaturation (95°C for 10 min), denaturation (95°C for 15 s), annealing and extension (60°C for 60 s) for a total 40 cycles. GAPDH was used as an internal control. mRNA levels are presented as: $2^{-\Delta\Delta Ct}$ (with Ct being the cycle threshold), where ΔCt= [Ct (Target gene)-Ct (GAPDH)].

2.4. Western blot

Total protein was extracted with cell lysis buffer (Pierce; Thermo Fisher Scientific) containing protease and phosphatase inhibitors. The concentration of total protein was detected using a bicinchoninic acid assay. A total of 30 μg protein was separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membrane. The membranes were blocked with 0.5% skimmed milk for 2 h at room temperature and then probed with primary antibodies overnight at 4°C, followed by 1 h incubation with the anti-rabbit IgG HRP-conjugated secondary antibodies (1:5,000) or anti-mouse IgG HRP-conjugated secondary antibodies (1:5,000) at room temperature. Subsequently, using TBST to wash the membranes, the immunoreactive bands were visualized using enhanced chemiluminescenc. Images were taken by the ChemiDoc XRS + system (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. RhoA GTPase assay

RhoA GTPase activity in cells was assessed using a RhoA G-LISA kit (Cytoskeleton, Denver, CO, USA) following the manufacturer's protocol. Briefly, cell lysates were prepared, and the protein concentration in each lysate was determined. Lysis buffer was added to equalize the protein concentrations across all samples. The prepared samples were then incubated in the provided wells and processed according to the instructions provided in the technical guide. The absorbance at 490 nm was measured using a microplate spectrophotometer.

2.6. Cell viability analysis

Cells of logarithmic growth stage were digested, centrifuged to remove supernatant, and resuspended in culture medium to prepare cell suspensions for counting. 4000 cells were inoculated in each well of the 96-well plate. After the cells were plastered, the culture medium was aspirated and discarded at different time points. Then, 100 μL containing 10% CCK-8 solution was added and incubated for 2 h at 37°C in an incubator. The absorbance values at 450 nm were measured using an enzyme marker. The data were analyzed and the activity curves of each group of cells were plotted.

2.7. Cell apoptosis assay

For apoptosis assays, cells with different treatments were incubated for 48 h. Following flow cytometry analysis (CytoFlex S, Beckman Coulter, CA, USA), the sample was labeled using Annexin-V-FITC/PI staining (BD Pharmingen, New Jersey, USA). Annexin V-positive cells were considered apoptotic and were examined using FlowJo (Ashland, OR, USA). The experiment was performed three times, and every concentration point was tested in duplicate.

2.8. Transwell migration and invasion analysis

Invasion assay: transwell chambers with matrigel were removed from the -20°C refrigerator and rewarmed at room temperature. After rewarming, 200 μL PBS was added to the transwell chambers and incubated at 37°C with 5% CO_2 for 1h. Then, DMEM medium containing 10% FBS was added to the lower chamber. 30,000 cells were inoculated into the lower chamber, and serum-free medium was used in the upper chamber. After inoculation, cells were incubated for 48 h. The original medium was poured off, and the lower chamber was fixed in 4% paraformaldehyde for 30 min. After fixation was completed, the PBS was rinsed 2-3 times. After the washing was completed, the lower chamber was placed in 0.1% crystal violet staining solution for 30 min. Then the crystalline violet staining solution was aspirated from the wells and rinsed 5 times repeatedly with water. The cells remaining in the chamber were wiped off with cotton swabs and then dried in a ventilated place. The pictures were observed and taken under the microscope. The migration experiment was performed as the invasion experiment except that matrigel was not applied and the migrated time is 24 h.

2.9. Immunofluorescence (IF) and F-actin visualization

Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 20 min at 4°C, and then blocked with 5% BSA in PBST for 30 min at room temperature. To visualize F-actin, cells were stained with Rhodamine-phalloidin (UElandy) for 20 min at room temperature. Nuclei were counterstained with DAPI for 10 min. The stained cells were observed using confocal microscopy, capturing images through confocal laser scanning. The acquired images were analyzed using ZEN software, and the fluorescence intensity was quantified using Image J software.

2.10. Stable-infected cell line establishment and in vivo tumorigenic assay

The lentiviral vectors used for stable transfection were purchased from GKN Shanghai, including sh-NC and sh-LTBP1. Logarithmic growth phase cells in a good growth state were digested, centrifuged, and counted one day before transfection. The cells were inoculated in culture plates at a concentration of 5×10^4 cells/well, and the culture plates were placed in a cell culture incubator containing 5% CO_2 at 37°C. After 24 h, the cells were observed and transfected when the cells grew to about 30%. After the medium was aspirated and discarded, 0.5 ml of lentiviral transfection solution was added. The cells were continued transfected for 48 h. When the density of adherent cells approached 25%, the cells were cultured with medium containing puromycin at a concentration of 2.5 μg/ml. The medium containing puromycin was replaced with new medium every 2 - 3 days. After 7 - 10 days of continuous culture, the cells were gathered for transfection efficiency testing in preparation for subsequent experiments. Male nude mice between the ages of 4 - 6 weeks were stochastically separated into 2 groups, each with 3 mice, housed in SPF environment. Stably-transfected cell lines in good condition were selected, digested and counted. The cells were inoculated in a certain number (1×10^6) in the right axilla of mice. After that, nude mice were continued to be reared for 2 - 3 weeks to observe the growth of subcutaneous tumors. The size of tumors was recorded periodically. After 5 weeks of rearing, the nude mice were executed to remove the tumors. The size of the tumor was measured and the weight of the tumor was weighed. The volume of the tumor was calculated (volume =

long diameter × short diameter^2/2) and the growth curve of the tumor was plotted.

2.11. Statistical Analysis

All data were expressed as mean ± standard deviation. Data were analyzed using SPSS22.0 software (IBM, Chicago, USA). Inter-group differences in normally distributed data were assessed for significance using one-way ANOVA followed by the LSD test. Differences associated with $p < .05$ were considered statistically significant.

3. Results

3.1. LTBP1 is highly expressed in and negatively correlated with the survivals of breast cancer patients

We firstly investigated the mRNA expression level of LTBP1 in paired breast cancer and normal adjacent tissues through online dataset analysis (TNMplot: differential gene expression analysis in Tumor, Normal, and Metastatic tissues) [9]. It was found that LTBP1 mRNA was highly expressed in breast cancer tissues compared with that of normal adjacent tissues (Figure 1A). Additionally, the correlation between LTBP1 mRNA expression and different types of survivals of breast cancer patients was analyzed through Kaplan-Meier Plotter [10]. We found that LTBP1 mRNA expression was negatively correlated with the relapse-free survival (RFS) (Figure 1B), distant-metastasis free survival (DMFS) (Figure 1C), and overall survival (OS) (Figure 1D) of breast cancer patients. Consistently, the protein level of LTBP1 was negatively correlated with the OS of breast cancer patients (Figure 1E). Notably, the mRNA expression of LTBP1 could be served as a predictive marker for the chemotherapy of breast cancer patients through ROC Plotter - Online ROC analysis, which indicated that LTBP1 mRNA expression was highly expressed in non-responders of chemotherapy (Figure 1F and 1G). These results demonstrate that LTBP1 might facilitate the progression of breast cancer progression.

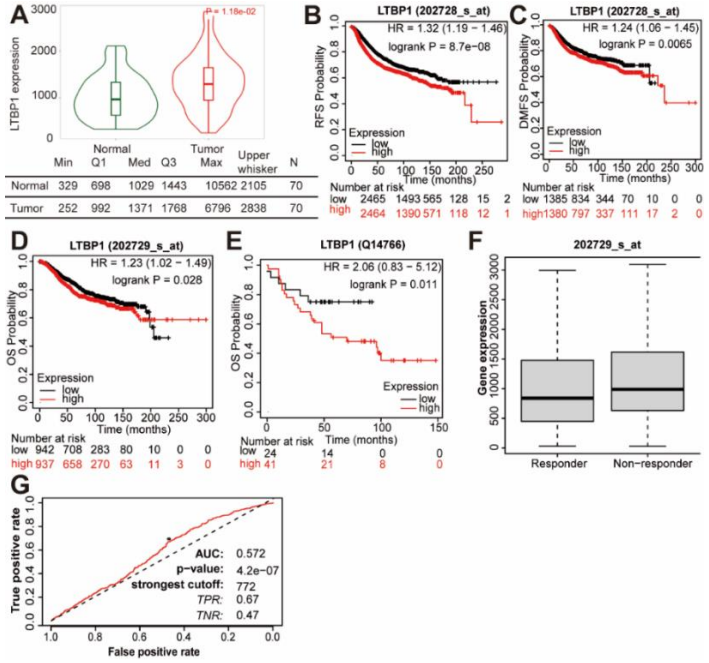

Figure 1. LTBP1 is highly expressed in and negatively correlated with the survivals of breast cancer patients.

Notes: (A) LTBP1 mRNA level was examined in breast cancer and normal adjacent tissues through online dataset. (B) The correlation between LTBP1 mRNA expression and RFS was evaluated through online dataset. (C) The correlation between LTBP1 mRNA expression and DMFS was determined through online dataset. (D) The correlation between LTBP1 mRNA expression and OS was detected through online dataset analysis. (E) The correlation between LTBP1 protein expression and OS measured through online dataset. (F and G) The correlation between LTBP1 mRNA expression and chemotherapy sensitivity was analyzed through online dataset.

3.2. LTBP1 promotes the proliferation and inhibits the apoptosis of breast cancer cells

To investigate the roles of LTBP1 in breast cancer progression, we examined the expression levels of LTBP1 in different types of breast cancer cells. As shown in figure 2A and 2B, LTBP1 exhibited a higher level in breast cancer cells compared to that of normal mammary epithelial cells, especially in triple negative breast cancer (TNBC) cells, which were chosen for the research objects in this work. Then LTBP1 was knocked down in TNBC cells and the knockdown efficiency was confirmed by RT-qPCR and western blot analysis (Figure 2C and 2D). Functional analysis including cell viability and cell apoptosis was performed to determine the effects of LTBP1 in breast cancer cell progression. It was found that knockdown of LTBP1 significantly suppressed the proliferation of breast cancer cells (Figure 2E and 2F). Furthermore, LTBP1 knockdown promoted the apoptosis of breast cancer cells, as evident by the upregulation of apoptotic cell ratio (Figure 2G) and the expression of apoptotic markers (Cleaved PARP and Cleaved Caspase 3) (Figure 2H).

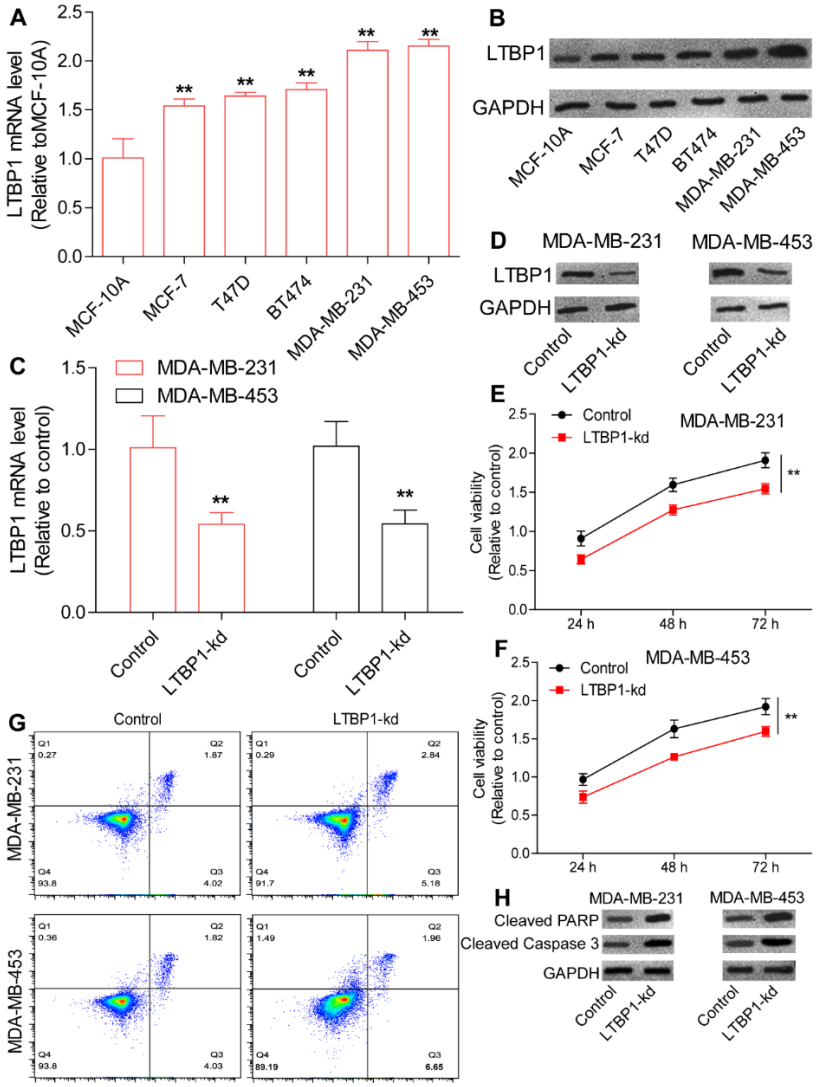

Figure 2. LTBP1 promotes the proliferation and inhibits the apoptosis of breast cancer cells.

*Notes: (A) LTBP1 mRNA level was detected in several breast cancer cell lines and mammary epithelial cells. (B) The protein level of LTBP1 was examined in several breast cancer cell lines and mammary epithelial cells. (C and D) The mRNA and protein levels of LTBP1 were measured in TNBC cells with or without LTBP1 knockdown. (E and F) Cell viability was determined in TNBC cells with or without LTBP1 knockdown. (G) Cell apoptosis was evaluated in TNBC cells with or without LTBP1 knockdown. (H) The protein levels of apoptotic markers were evaluated in TNBC cells with or without LTBP1 knockdown. **P < 0.01 vs control.*

3.3. LTBP1 promotes the metastatic ability of breast cancer cells

Since most of the death of breast cancer patients is resulted by the metastasis, we further evaluated the effects of LTBP1 on the metastatic ability of breast cancer cells. As shown in figure 3A – 3D, transwell-migration analysis revealed that LTBP1 knockdown remarkably attenuated the migrated ability of TNBC cells. In consistent, the invaded ability of TNBC cells was suppressed by LTBP1 knockdown (Figure 3A – 3D). Furthermore, we explored whether LTBP1 was involved in the Epithelial-mesenchymal transition (EMT) process of TNBC cells as EMT acts as a critical promoter during breast cancer metastasis. We found that LTBP1 knockdown significantly inhibited the EMT process in TNBC cells, as evident by the upregulation of epithelial marker expression and downregulation of mesenchymal marker expression (Figure 3E – 3G). These results indicate that LTBP1 serves as a tumor-promoter in breast cancer cell progression.

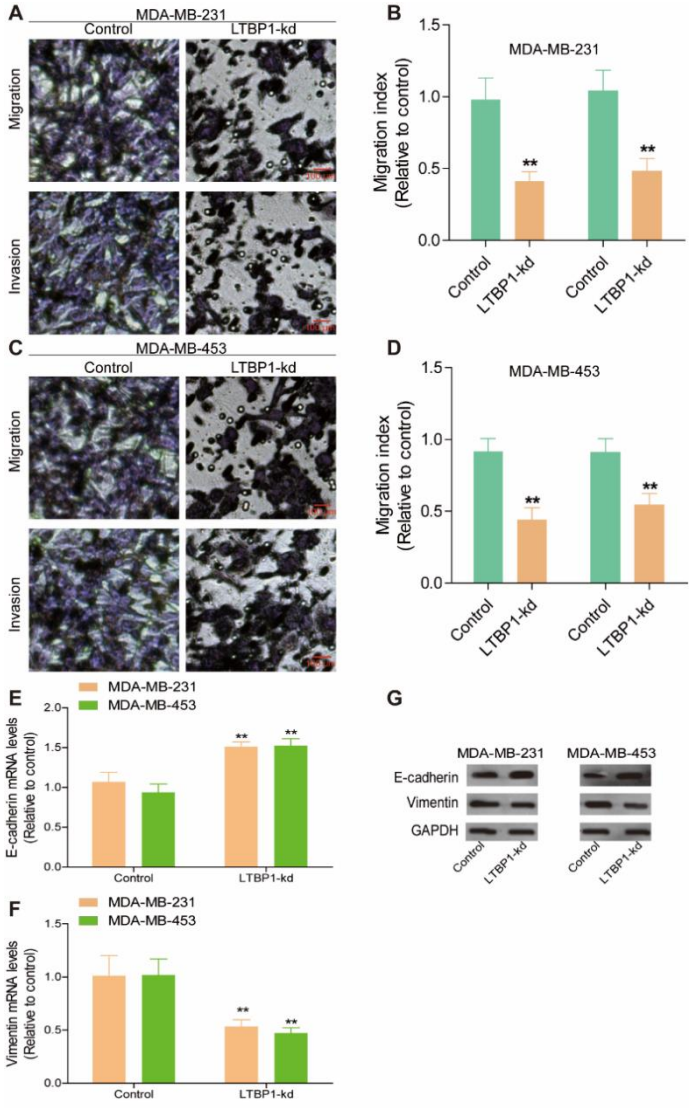

Figure 3. LTBP1 promotes the metastatic ability of breast cancer cells.

*Notes: (A and B) The migration and invasion ability of MDA-MB-231 cells with or without LTBP1 knockdown was determined through transwell migration and invasion analysis. (C and D) The migration and invasion ability of MDA-MB-453 cells with or without LTBP1 knockdown was measured through transwell migration and invasion analysis. (E) The mRNA level of E-cadherin was detected in TNBC cells with or without LTBP1 knockdown. (F) The mRNA level of Vimentin was examined in TNBC cells with or without LTBP1 knockdown. (G) The protein levels of E-cadherin and Vimentin were examined in TNBC cells with or without LTBP1 knockdown. **P < 0.01 vs control.*

3.4. LTBP1 facilitates the progression of TNBC cells in vivo

To validate the effects of LTBP1 on TNBC cell progression, we constructed TNBC cells with or without LTBP1-stable knockdown through lentivirus infection. The knockdown efficiency was confirmed by RT-qPCR and western blot analysis (Figure 4A and 4B). Then the effects of LTBP1 on tumor growth were detected by using the nude mouse xenograft model through subcutaneously inoculation. We identified that LBTP1 knockdown significantly suppressed the proliferation of TNBC cells by detecting tumor growth volume at different time-points (Figure 4C and 4D). Furthermore, the tumor weight was significantly reduced in tumors derived from TNBC cells with LTBP1 knockdown (Figure 4E). Consistently, the expression level of Ki67, a proliferation marker, was decreased in tumors derived from TNBC cells with LTBP1 knockdown (Figure 4F and 4G). Overall, there results suggest that LTBP1 can promote TNBC progression.

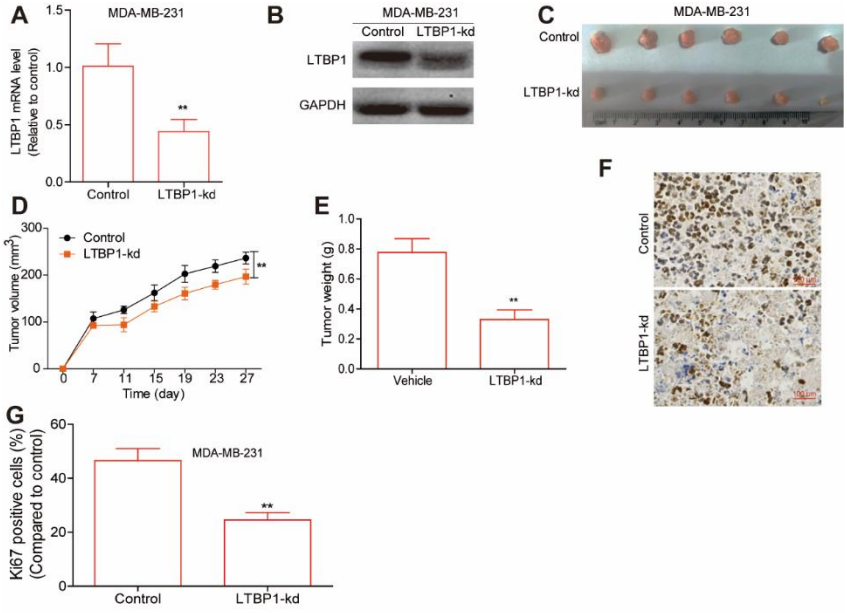

Figure 4. LTBP1 facilitates the progression of TNBC cells *in vivo*.

*Notes: (A) The mRNA level of LTBP1 was examined in MDA-MB-231 cells with or without the infection of LTBP1 knockdown lentivirus. (B) The protein level of LTBP1 was detected in MDA-MB-231 cells with or without the infection of LTBP1 knockdown lentivirus. (C) Tumor images derived from MDA-MB-231 cells with or without LTBP1 knockdown. (D) The volume was measured for the tumors derived from MDA-MB-231 cells with or without LTBP1 knockdown at different timepoints. (E) Tumor weight was determined for the tumors derived from MDA-MB-231 cells with or without LTBP1 knockdown. (F and G) Ki67 protein level was examined in tumors derived from MDA-MB-231 cells with or without LTBP1 knockdown. ** P < 0.01 vs control.*

3.5. LTBP1 activates the RhoA/ROCK signaling pathway in breast cancer cells

Then we explored the underlying mechanisms by which LTBP1 promotes TNBC progression. We firstly analyzed the genes with the expression positively correlated with LTBP1 in breast cancer through online datasets (TNMplot: differential gene expression analysis in Tumor, Normal, and Metastatic tissues) [9] (Supplementary Table 1). Then these genes were subjected to GO and KEGG enrichment analysis. As shown in figure 5A and 5B, it was shown that genes positively correlated with LTBP1 were enriched in in biological processes (BP) including regulation of signaling and organelle organization, cell metastasis and so on (Figure 5A), cellular components (CC) such as organelle membrane, adherens junction, and cell leading edge (Figure 5B), and molecular functions (MF) like enzyme binding and GTPase binding, small GTPase binding, and cell adhesion molecule binding (Figure 5C). According to KEGG analysis, the correlated genes were significantly associated with the MAPK, TNF, AMPK, cGMP-

PKG and Rap1 pathways (Figure 5D). Within the GTP enzyme family, Rho GTPases play a crucial role in various cellular processes such as cell migration, phagocytosis, contraction, and adhesion. Specifically, Rho proteins, including RhoA, are known to promote the formation and elongation of stress fibers, actin bundle contraction, and directional adhesion. The RhoA/ROCK signaling pathway, activated by the binding of RhoA and GTP, triggers downstream ROCK activation, leading to the phosphorylation of its substrates, such as Myosin Light Chain 2 (MLC2), which remodels the cytoskeleton and induces actin filament stabilization. To verify the effect of LTBP1 on the RhoA signaling pathway, we first investigated its impact on RhoA activity using a G-LISA RhoA activation assay. The results demonstrated that LTBP1 knockdown significantly inhibited RhoA activity in TNBC cells (Figure 5E). Furthermore, western blotting analysis revealed that LTBP1 knockdown significantly down-regulated the phosphorylation levels of MLC2 at the Ser19 position (Figure 5F). Consistently, IF analysis of F-actin using Rhodamine-Phalloidin staining revealed that anlotinib reduced the formation of F-actin in these cell lines (Figure 5G and 5H). These results suggest that LTBP1 knockdown can suppress the RhoA/ROCK signaling pathway.

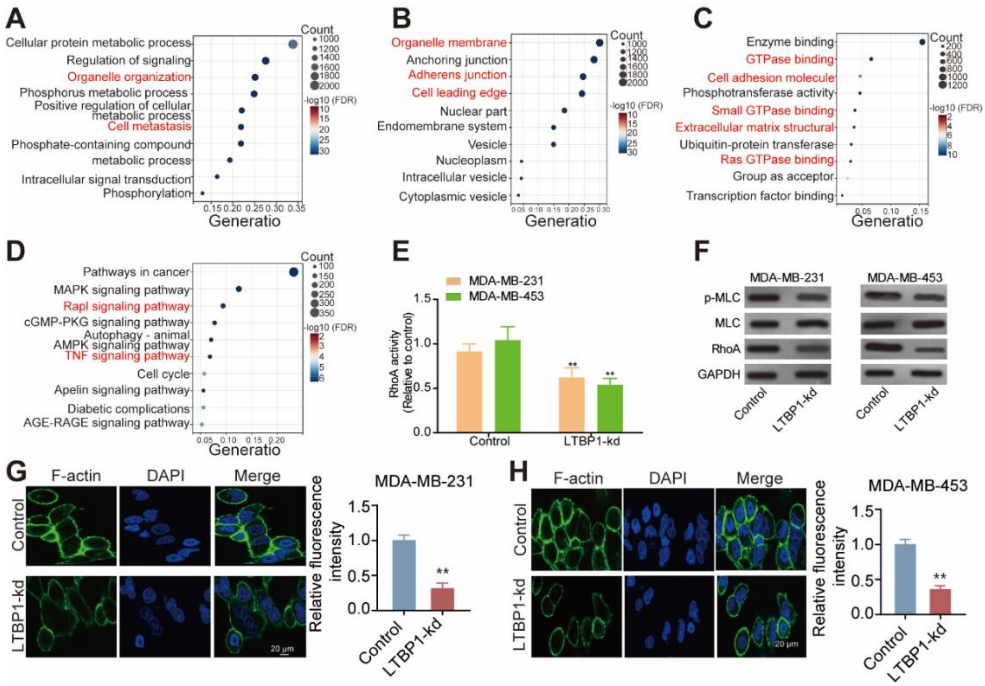

Figure 5. LTBP1 activates the RhoA/ROCK signaling pathway in breast cancer cells.

*Notes: (A - C) The genes positively correlated with were subjected to the GO enrichment analysis including the following: (A) Biological processes (BP), (B) Cellular components (CC) and (C) Molecular functions. (D) KEGG enrichment analysis of LTBP1 and its positively correlated genes in breast cancer. (E) RhoA activity was measured through G-lisa assay in TNBC cells. (F) The protein levels of p-MLC, MLC, RhoA were detected in TNBC cells with or without LTBP1 knockdown. (G and H) The formation of F-actin was evaluated in TNBC cells with or without LTBP1 knockdown. **P < 0.01 vs control.*

3.6. LTBP1 promotes TNBC progression dependent on the RhoA/ROCK signaling pathway

Finally, to determine whether the RhoA/ROCK signaling pathway was involved in LTBP1-mediated effects on TNBC cell progression, Narciclasine (Nar), the activator of the RhoA/ROCK signaling pathway was added in TNBC cells with or without LTBP1 knockdown. The activation efficiency of Narciclasine was confirmed by RhoA activity and western blot analysis (Figure 6A and 6B). Notably, activation of the RhoA/ROCK signaling pathway held no effect on LTBP1 expression in TNBC cells (Figure 6B). It was found that the activation of the RhoA/ROCK signaling pathway rescued the inhibitory effects of LTBP1 knockdown on the proliferation ability of TNBC cells (Figure 6C and 6D). Cell apoptosis revealed that the promoting effects of LTBP1 on TNBC cell apoptosis was attenuated by the

activation of the RhoA/ROCK signaling pathway (Figure 6E). Furthermore, transwell migration and invasion analysis obtained a consistent result, as evident by the rescuing of cell migration and invasion ability in cells treated with the activator of the RhoA/ROCK signaling pathway (Figure 6F – 6I). Besides, the EMT process suppressed by LTBP1 knockdown was reversed by Nar treatment (Figure 6J - 6L). Taken together, our results suggest that LTBP1 could serve as an oncogene for TNBC through the activation of the RhoA/ROCK signaling pathway.

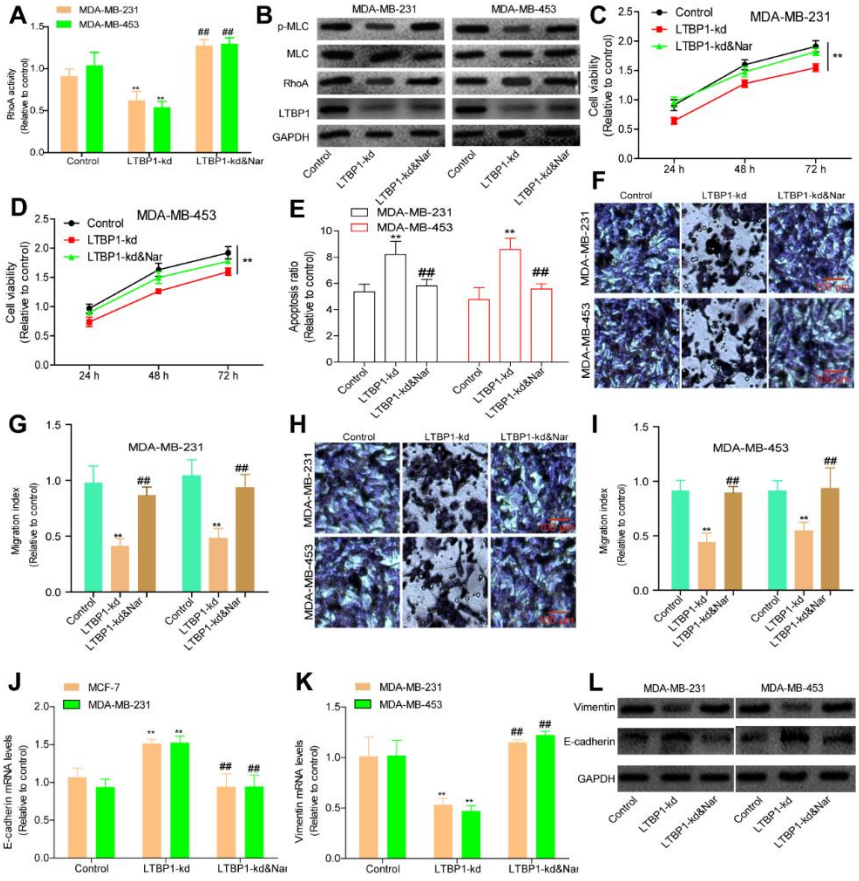

Figure 6. LTBP1 promotes TNBC progression dependent on the RhoA/ROCK signaling pathway.

*Notes: (A) RhoA activity was measured in TNBC cells with LTBP1 knockdown as well as Nar treatment or not. (B) The protein levels of p-MLC, MLC, RhoA, and LTBP1 were detected in TNBC cells with LTBP1 knockdown as well as Nar treatment or not. (C and D) Cell viability was examined in the cells depicted in (A). (E) Cell apoptosis was determined in cells described in (A). (F - I) The migration and invasion ability was evaluated in cells depicted in (A). (J - L) The expression of EMT markers was detected in cells described in (A). **P < 0.01 vs control; ##P < 0.01 vs LTBP1-kd group.*

4. Discussion

The present study unveils a novel role for LTBP1 in the progression of triple-negative breast cancer (TNBC) and sheds light on its potential implications as a therapeutic target. Our findings demonstrate that elevated LTBP1 expression is associated with adverse clinical outcomes, including RFS, DMFS, and OS in breast cancer patients. Moreover, LTBP1 expression correlates with resistance to chemotherapy, suggesting its potential as a predictive biomarker for treatment response. Notably, LTBP1 exhibits a higher level in TNBC cells than that of other cell types, which hints the positive correlation between LTBP1 expression and tumor progression as TNBC displays the worst type of breast cancer [12]. Indeed, the functional assays conducted in TNBC cell lines support a pivotal role for LTBP1 in promoting tumorigenesis. LTBP1 knockdown results in a significant reduction in cell proliferation, accompanied by an induction of apoptosis. These observations are consistent with the negative correlation

observed between LTBP1 expression and patient survival outcomes, highlighting the potential clinical relevance of LTBP1 as a prognostic marker in TNBC.

Importantly, our *in vitro* findings also reveal that LTBP1 plays a critical role in the metastatic behavior of TNBC cells. LTBP1 knockdown attenuates cell migration and invasion, crucial steps in the metastatic cascade [13]. These results are particularly noteworthy given that metastasis is a major contributor to the high mortality associated with TNBC. The inhibition of EMT further supports the antimetastatic effects of LTBP1 knockdown, suggesting a broader impact on the invasive properties of TNBC cells. And this effect is consistent the previous study showing that LTBP1 promotes esophageal squamous cell carcinoma progression through EMT and cancer-associated fibroblasts transformation [14]. Future studies can be performed on the effects of LTBP1 on the progression of other types.

Mechanistically, our study implicates the RhoA/ROCK signaling pathway as a downstream effector of LTBP1 in TNBC. The positive correlation between LTBP1 and genes involved in this pathway suggests that LTBP1 may activate RhoA, leading to downstream ROCK activation. Supporting this hypothesis, LTBP1 knockdown results in decreased RhoA activity and reduced phosphorylation of Myosin Light Chain 2 (MLC2), indicating suppression of the RhoA/ROCK signaling axis. Importantly, the RhoA/ROCK signaling pathway has been shown to play crucial roles in tumor metastasis by large number of studies [15-17]. Our provides a mechanistic insight into how LTBP1 may modulate cellular processes associated with tumorigenesis, including cytoskeletal reorganization and cell motility through the RhoA/ROCK signaling pathway. In the context of *in vivo* studies, our xenograft mouse model demonstrates that LTBP1 knockdown significantly impedes TNBC tumor growth. Tumor weight and Ki67 expression, a marker of proliferation, are notably reduced in LTBP1-deficient tumors [18]. This aligns with our *in vitro* observations and strengthens the argument for LTBP1 as a potential therapeutic target in TNBC.

The therapeutic implications of our findings are underscored by the identification of the RhoA/ROCK signaling pathway as a downstream mediator of LTBP1. The rescue experiments using the RhoA/ROCK pathway activator, Nar, provide evidence that the effects of LTBP1 knockdown on cell proliferation, apoptosis, migration, and invasion are partially reversed upon pathway activation. Notably, Nar has been demonstrated to hold anti-tumor and anti-inflammatory properties [19, 20]. Additionally, Nar can attenuate sepsis-induced myocardial injury by modulating autophagy [21]. This suggests that targeting the RhoA/ROCK axis may be a potential strategy for mitigating the oncogenic effects of LTBP1 in TNBC. Furthermore, suppression of LTBP1 was recently shown to attenuate natural killer/T cell lymphoma progression by inactivating the TGF-β/Smad and p38MAPK pathways, which suggests us that LTBP1 might regulate the immunotherapy in TNBC that was weakly responded to immunotherapy currently [22].

In conclusion, our study establishes LTBP1 as a significant player in TNBC progression, impacting cell proliferation, apoptosis, and metastasis through modulation of the RhoA/ROCK signaling pathway. These findings contribute valuable insights into the molecular mechanisms underlying TNBC pathogenesis and provide a basis for further exploration of LTBP1 as a therapeutic target in this aggressive breast cancer subtype. Future studies elucidating the precise interactions between LTBP1 and the RhoA/ROCK pathway may uncover additional avenues for targeted therapeutic interventions, ultimately improving the clinical management of TNBC.

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Conflict of interest

All the authors claim that the manuscript is completely original. The authors also declare no conflict of interest.

Author contributions

Conceptualization: Jun Wang; Investigation: Jingcheng Zhang; Methodology: Hong Deng; Formal analysis: Jingcheng Zhang; Writing – original draft: Jingcheng Zhang, Hong Deng; Writing – review & editing: Jun Wang.

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